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14. ABSTRACT Prostaglandin E2 is a powerful chemical that can cause an increase in blood stem cells. The theory that many cancers, including breast cancers, also contain cancer stem cells is being studied. Cancer stem cells are thought to be resistant to some types of cancer treatments, and allow tumors to re-grow and possibly to spread after cancer treatment. If this theory is true, understanding how breast cancer stem cells live and grow may be an important breakthrough to developing new, more effective treatments for breast cancer. This study showed that prostaglandin E2 caused an increase in breast cancer stem cells in several cell lines, identified the prostaglandin E receptor EP2 as the active receptor and determined the signaling pathway for prostaglandin E2 stimulation. These results suggest that drugs targeting the prostaglandin E2 receptor or the signaling pathway could be new ways to attack and reduce a breast cancer stem cell-like population.					
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1. Introduction

Prostaglandin E2 is a powerful biomolecule with many reported functions (1,2). One of the enzymes that catalyze the production of prostaglandin E2 is cyclooxygenase 2 (COX2). COX2 expression in breast cancer cells in human samples has been associated with poor outcome, including shorter survival (3). However, the exactly how COX2 and prostaglandin E2 contribute to worse outcomes in breast cancer is not known. Prostaglandin E2 can promote angiogenesis, inhibit immunity, increase invasion, and promote cell division (1,2). However, prostaglandin E2 may influence breast cancers in other ways as well. This proposal arose from two independent observations: first, studies that many cancers, including breast cancers, contain a “cancer stem cell” population that is resistant to chemotherapy and is responsible for tumor recurrence and metastasis (4), and second, the observation that prostaglandin E2 can expand hematopoietic stem cell populations (5). The objective of this study was to determine if prostaglandin E2 treatment would also increase breast cancer stem cell populations *in vitro*.

2. Body

This project was to determine whether prostaglandin E2 (PGE2) stimulation increased the breast cancer stem cell population, using two main assays, the enzymatic Aldefluor (ALDH) assay, and a “mammosphere” sphere-forming assay in defined medium in low adherence culture plates. The work was divided as described in the Statement of Work:

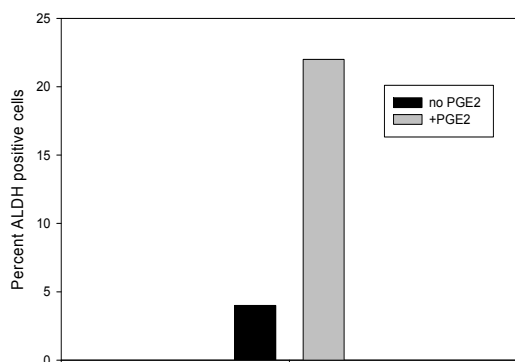
Task 1. Evaluation response to PGE2 for aldehyde dehydrogenase (ALDH) positive populations

The first goal of the project was to obtain four human breast cancer cell lines (Sum 149, Sum159, MCF7 and SKBr3) and verify their unique identities using DNA fingerprinting (6). A number of recent reports have demonstrated misidentification and contamination in cell lines (6), and since we are relatively new to the culture of breast cancer cell lines, we wanted to verify any lines that would be used in the study. We initially attempted to perform the DNA fingerprinting at the University of Michigan DNA sequencing core, but found that the analysis was not acceptable. We therefore used a commercial site (RADIL at the University of Missouri) to perform the reactions and analysis. There was some delay because of the problems with fingerprinting, but we did not want to proceed with analysis if the cells had not been verified. We were able to confirm that the Sum149 and Sum159 were unique. After testing MCF7 cells from a variety of sources, we concluded that the cells were either misidentified or contaminated. Consequently, MCF7 was obtained from ATCC which confirms the DNA profile of each cell line. SkBr3 was obtained from a collaborator (M. Wicha, University of Michigan). However, preliminary results with SkBr3 revealed that the ALDH studies were not useable (this cell line has very high positive levels, so increases cannot be detected). We therefore continued with analysis using the three cell lines.

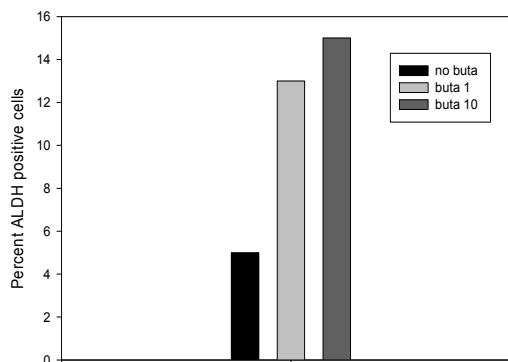
Next, we determined if PGE2 stimulation resulted in increased in an ALDH-positive (putative breast cancer stem cell) population. The method has been previously described (4). These studies revealed that MCF7 was ALDH negative, and no positive population was detected with PGE2 stimulation. Sum149 showed no consistent changes with PGE2 stimulation. Sum159 cells show increased of up to 400% in ALDH positive cells. Additionally, the prostaglandin receptor EP2 agonist butaprost (dose responsive at 1 and 10 μ M) also caused an increase in Sum159 ALDH positive population, suggesting that the EP2 receptor was involved in the ALDH positive population increases (7). Results are shown in Figure1A and B. Note: the results were reproducible in multiple experiments but not performed with enough replicates to perform statistical analyses.

Figure 1 Aldefluor (ALDH) Assay with PGE2 or Butaprost in Sum 159 Breast Cancer Cells**A. PGE2 (0 or 1uM)**

Sum159 Aldefluor Assay with PGE2

**B. Butaprost (0, 1 or 10 uM)**

Sum159 Aldefluor Assay with Butaprost

**Task 2. Response of mammosphere culture to PGE2 treatment**

The next goal was to evaluate the effect of PGE2 in a second breast cancer stem cell assay, the “mammosphere” formation assay. This assay tests the ability of cells to form organoid spheres in defined, serum free medium in low adherences dishes. Cells that can proliferate as spheres are anchorage independent, which is a recognized property of “stem” type cells (8). The assay is normally performed in a serum-free defined medium supplemented with insulin and epidermal growth factor (EGF). We performed the assay and observed increases in Sum159 mammospheres in this complete medium, while Sum 149 showed very modest increases and MCF7 did not show increases. To test whether PGE2 could support mammosphere formation in a less supportive culture medium, we also performed the test in medium lacking EGF and/or insulin. Sum 149 and MCF7 cells showed significant increases in mammosphere formation when tested in medium without insulin or without EGF (shown in Figure 3A and B without EGF). These data suggest that all three cell lines can respond to PGE2 stimulation with an increase in the breast cancer stem cell population, but that a more restrictive medium is needed to observe the change in Sum 149 and MCF7 cells. We hypothesize that PGE2 is replacing the growth signal normally received through EGF in Sum 149 and MCF7 cells. Note: the results were reproducible in multiple experiments but not performed with enough replicates to perform statistical analyses.

**Figure 2 Sum159 Sphere Assay
(Complete medium with 0 or 1 uM PGE2)**

Sum159 Sphere Assay

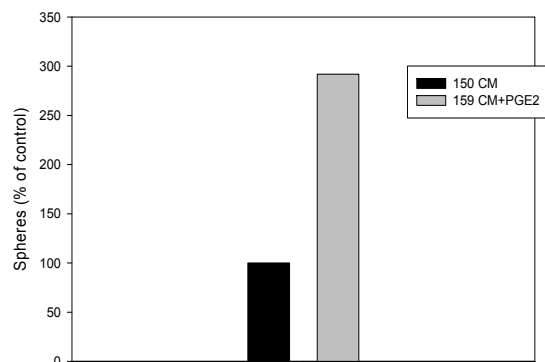
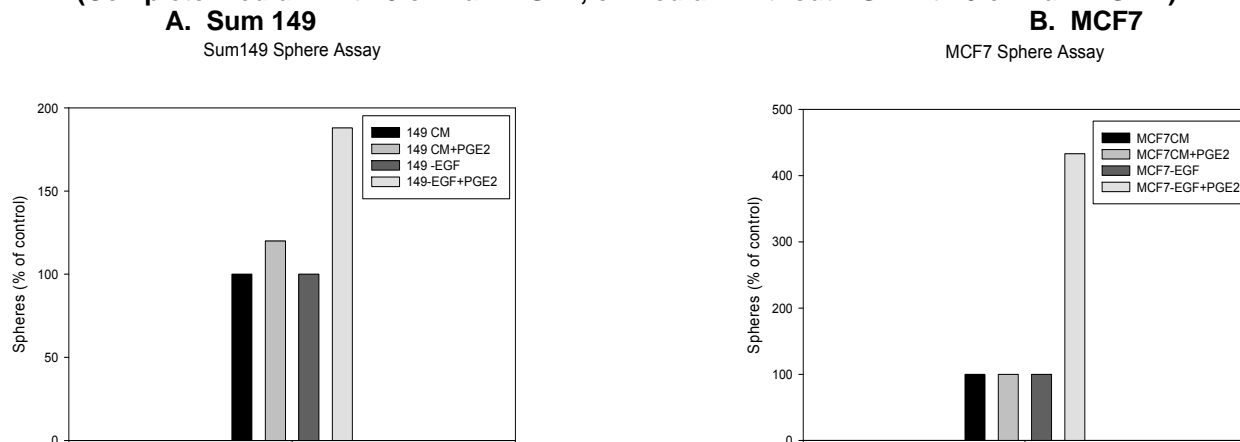


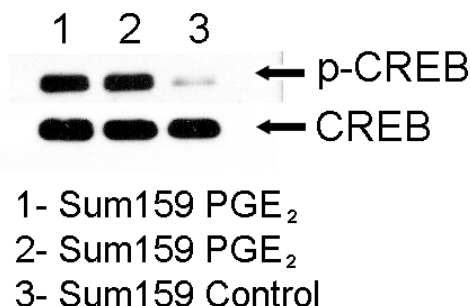
Figure 3 Sum149 and MCF7 Sphere Assays
(Complete medium with 0 or 1 uM PGE₂, or medium without EGF with 0 or 1 uM PGE₂)



Task 3. Characterize genetic and protein signature of PGE₂ response

The final goal was to determine the signaling pathways responsible for PGE₂ expansion of breast cancer stem cell populations. We had originally planned to use expression arrays to evaluate the mRNA responses to PGE₂. However, based on the assay results, we suspected that protein phosphorylation would provide better insight into the processes. We screened Sum149 and Sum159 for cell signaling using protein phosphorylation antibody array (Proteome Profiler, R&D Systems). In response to PGE₂ stimulation, we observed an increase in phospho-CREB after stimulation. This signaling is typical for responses through the EP2 receptor through cAMP/PKA (9). These data indicate that PGE₂ stimulation can cause increases in the breast cancer stem cell population in Sum159 cells. The ability of PGE₂ to replace insulin or EGF suggests that PGE₂ stimulation may stimulate pathways downstream of EGF and/or insulin (thus “short circuiting” the need for EGF). To test this hypothesis, we plan future experiments using inhibitors of those pathways (e.g., PD98059 or U0126 for the ERK/MEK pathway). If the signaling occurs through cAMP/PKA, as indicated by the p-CREB results noted in the previous experiments, inhibitors of PKA (e.g., H89) should also “short circuit” the formation of mammospheres in MCF7 and Sum149 cells cultured without EGF but supplemented with PGE₂. We are also using a new immunostaining assay (PathScan multiplex kit, Cell Signaling Technologies) to determine if PGE₂ stimulation results in EGF signaling through ERK1/2 phosphorylation.

Figure 4. Changes in CREB Phosphorylation with PGE₂ Stimulation in Sum 159 Breast Cancer Cells.



3. Key Research Accomplishments

- Prostaglandin E₂ treatment caused expansion of breast cancer stem-like cell populations in several human breast cancer cell lines

- Treatment with the prostaglandin E2 reception agonist butaprost resulted in nearly complete replication of the prostaglandin E2 treatment, suggesting that the action is mediated by the prostaglandin E2 EP2 receptor
- Mammosphere assays indicated that stimulation with prostaglandin E2 was not detected unless the growth medium was devoid of additional epidermal growth factor and insulin, suggesting that prostaglandin E2 acts through cellular signaling pathways also used by insulin and/or epidermal growth factor
- Phosphorylation studies showed a large increase in CREB signaling following prostaglandin E2 stimulation, indicating that the primary signaling for prostaglandin E2 is through PKA

4. Reportable Outcomes

- Series of breast cancer cell lines with DNA verification
- New methodology for mammosphere production without epidermal growth factor and insulin

5. Conclusion

We have shown that PGE2 stimulation can cause increases in the breast cancer stem cell populations in three breast cancer cell lines, and are closing in on the mechanism of action. If our working hypotheses are correct, then treatments focused on preventing the generation of PGE2 (COX2 inhibitors) or interrupting the signaling pathways downstream of PGE2 and/or EGF (such as ERK1/2 inhibitors) could be used therapeutically for improved outcomes in women with breast cancer. Since such treatments would target the breast cancer stem cell populations, they should be used in addition to agents targeting the entire breast cancer populations in a neoadjuvant setting.

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